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Novel synthetic collagen fibers, poly(PHG), stimulate platelet aggregation through glycoprotein VI

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ABSTRACT

Novel synthetic collagen fibers, poly(PHG) made by polycondensation of Pro-Hyp-Gly, spontaneously assume polymeric structure with molecular weights greater than 10^5. Its application for biomaterials has been explored, but that for a platelet agonist has not been investigated. Poly(PHG)-induced platelet aggregation independently of thromboxane A_2 and integrin α2β1. Poly(PHG)-induced tyrosine phosphorylation of glycoprotein VI (GPVI)-related molecules and failed to activate GPVI/FcRγ-deficient platelets. Binding of GPVI to poly(PHG) was confirmed by a surface plasmon resonance spectroscopy, suggesting that poly(PHG) activates platelets through GPVI. Poly(PHG) is an useful research tool to investigate GPVI-mediated signals and a substitute for collagen in platelet functional assays.

1. Introduction

The interaction between platelets and collagen exposed at sites of vessel damage plays an important role not only in cessation of bleeding but also in pathological thrombus formation that is responsible for stroke and myocardial infarction. The interaction between collagen receptors in platelets and collagen leads to the inside-out activation of the integrin αIIbβ3 and the release of the secondary mediators, such as ADP and thromboxane A2 (TXA2), culminating in platelet aggregation mediated by fibrinogen binding to αIIbβ3 and thrombus formation.

In clinical practice, platelet functional tests are often performed to evaluate bleeding disorders or to monitor the effect of antiplatelet drugs. Platelet functions are ordinarily assessed with platelet aggregometry based upon changes in the optical density of platelet suspensions when stimulated with platelet activators (agonists). For this purpose, collagen fibers purified from animal tissues are widely used as an agonist. However, collagen preparations derived from animal tissues have several shortcomings, such as variability in biological activities and susceptibility to contamination by infectious pathogens. Particularly, the former is one of the largest hindrances to preclude the standardization of platelet aggregation tests in clinical use.

Platelets are known to have two major receptors for collagen, the integrin α2β1 and the glycoprotein VI/FcRγ-chain complex (GPVI), as well as with a number of minor receptors of uncertain significance [1]. Since GPVI is the most powerful signaling receptor among collagen receptors that plays a major role in thrombus formation [1], the GPVI signal transduction pathway has been the target for extensive investigation. To define the characteristics of the GPVI-mediated platelet activation, researchers have conventionally used GPVI specific agonists, collagen-related peptide (CRP; Gly-Lys (or Cys)-Hyp-(Gly-Pro-Hyp)$_{10}$-Gly-Lys (or Cys)-Hyp-Gly) [2,3] and the snake toxin convulxin [4]. However, the experiments using these agents have often met difficulties, since CRP must be cross-linked in each laboratory, which results in variable activity, and convulxin easily undergoes inactivation.

Recently, Tanihara and his group developed synthetic collagen fibers consisting of the Pro-Hyp-Gly sequence by direct polycondensation of Pro-Hyp-Gly tripeptides [5]. The synthetic collagen fibers named as poly(PHG) have molecular weights greater than 10^5 and form triple-helical structure like natural collagen. Similar to natural collagen, poly(PHG) is a huge polypeptide and spontaneously assumes polymeric structure without chemical cross-linking.

Abbreviations: GP, glycoprotein; LAT, linker for activation of T cells; SLP-76, SH2 domain-containing leukocyte protein of 76; PATI, platelet aggregation threshold index; PLCγ2, phospholipase Cγ2; TXA2, thromboxane A2.

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Since poly(PHG) has collagen-like structure, but is thermally stable up to the temperature of 80 °C and it contains no pathogen, application of poly(PHG) for biomaterials and cosmetics has been extensively investigated [6]. However, whether poly(PHG) can be used as a platelet agonist has not been elucidated to date.

In this study, we demonstrate that poly(PHG) induces platelet aggregation by binding to the collagen receptor GPVI more potently than CRP or collagen and suggest that poly(PHG) serves as a better GPVI agonist as a research tool compared with CRP or convulxin in terms of its high potency in inducing platelet aggregation,

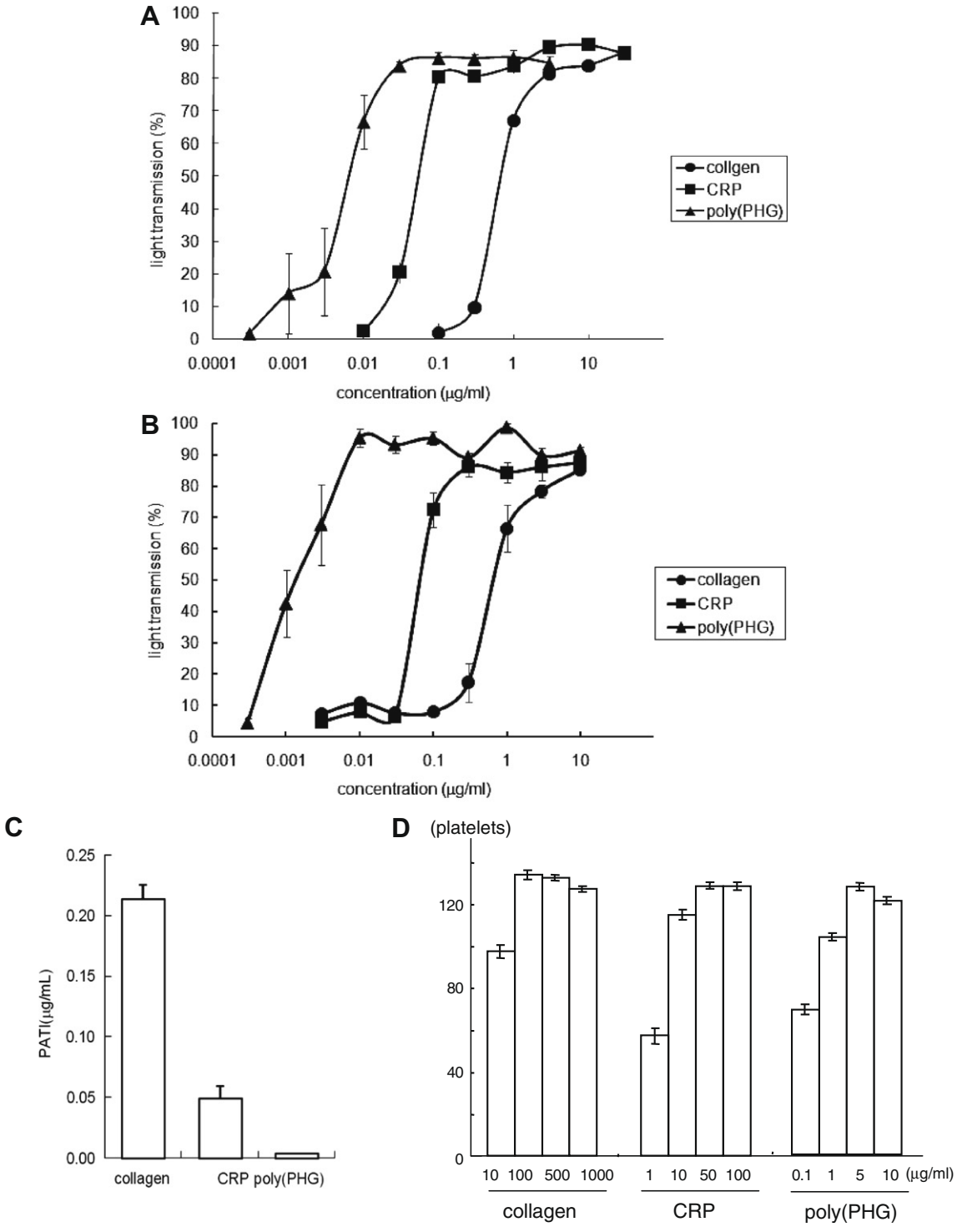

Fig. 1. Poly(PHG), CRP, and collagen dose-dependently stimulates platelet aggregation and adhesion. (A and B) Human washed platelets (A) or PRP (B) were stimulated with the indicated concentrations of poly(PHG), CRP, or collagen. The data are expressed as the means ± S.E. (n = 3–8 from three experiments). (C) Whole blood was stimulated with four concentrations of collagen, CRP, or poly(PHG), and the minimum concentration of an agonist that induces 50% aggregation (PATI: platelet aggregation threshold index (μg/ml)) was calculated. The data are expressed as the means ± S.E. (n = 7 from two experiments). (D) Washed human platelets were seeded on cover slips coated with the indicated concentrations of collagen, CRP or poly(PHG) for 30 min. Adherent platelets were fixed, permeabilized, stained, and visualized as described in Section 2. The graph illustrates the mean number of adhered platelets ± S.E. per 0.022 mm^2 (n = 8–20 from two experiments).

cost, and stability. Poly(PHG) can also be used as an agonist for platelet aggregation test in clinical use.

2. Materials and methods

2.1. Materials

Fc Receptor (FcR) γ-chain-deficient mice (C57BL/6) were generous gifts from Dr. Takashi Saito (RIKEN Research Center for Allergy and Immunology, Japan). The recombinant extracellular domain of human GPVI expressed as a dimeric human immunoglobulin Fc domain fusion protein (GPVI-hFc2) was kindly donated by Drs. Masaaki Moroi and Yoshiki Miura (Kurume University, Japan) [7]. Collagen reagent Horm, as native type I fibrils from equine tendons, was from Nycomed Pharma (Munich, Germany). CRP (GKO[GPO]10GKOG; single-letter code, where O is hydroxyproline) was synthesized by Toray Research Center (Tokyo, Japan); it was cross-linked with 0.25% glutaraldehyde for 3 h at 4 °C and then dialyzed into phosphate-buffered saline (PBS). Poly(PHG) was synthesized as described previously [5]. In brief, Pro-Hyp-Gly (Peptide Institute, Osaka, Japan) was dissolved in phosphate buffer and mixed with 1-hydroxybenzotriazole and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride. After stirring for 2 h at 4 °C, and then for 46 h at 20 °C, the reaction mixture was dialyzed against water. A glutathione *S*-transferase (GST)-fusion protein containing the tandem SH2 domains of Syk (GST-Syk-SH2) was obtained and expressed as described previously [8]. The recombinant human immunoglobulin Fc domain fusion protein (hFc2) was produced as described previously [9]. The following materials were obtained from the indicated suppliers, respectively: phospholipase Cγ2 (PLCγ2) (Santa Cruz Biotechnology, CA); anti-LAT (linker for activation of T cells) antibody, anti-phosphotyrosine antibody (4G10), and anti-integrin α2 antibody (A2-IIE10) (Millipore Corporation, MA, USA); anti-SLP-76 (SH2 domain-containing leukocyte protein of 76) antibody (Merck, Darmstadt, Germany); anti-Syk antibody (Wako, Osaka, Japan); All other reagents were from previously described sources [10].

2.2. Platelet preparation

This study was approved by the Ethical Committees in University of Yamanashi and informed consent was provided according to the Declaration of Helsinki. Venous blood from healthy drug-free volunteers was collected into 10% sodium citrate (3.8% sodium citrate, w/v). Washed human platelets resuspended in modified Tyrode's buffer (137 mM NaCl, 11.9 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 1.1 mM $MgCl_2$ and 5.6 mM glucose, pH 7.3) (2×10^8/ml for aggregation study, 2×10^7/ml for adhesion study, 1×10^9/ml for immunoprecipitation) were obtained as described elsewhere [10]. Murine blood was drawn and washed platelets (2×10^8/ml) were obtained as described elsewhere [10].

2.3. Platelet aggregation

Washed platelets or platelet-rich plasma (PRP) were stimulated with indicated concentrations of collagen, CRP, thrombin, TXA2 analogue U46619, or poly(PHG) and aggregation was monitored by a PA-100 platelet aggregation analyzer (Kowa, Tokyo, Japan). Where indicated, platelets were pretreated with the indicated concentrations of A2-IIE10, hFC2, GPVI-hFc2, DMSO, or 10 μM indomethacin at 37 °C for 5 min. Whole blood was also stimulated by four different concentrations of collagen, CRP or poly(PHG), and platelet aggregation was measured by WBA-Neo according to the manufacturer's instructions (ISK, Tokyo, Japan). The minimum concentration of an agonist that induces 50% aggregation was determined as the platelet aggregation threshold index (PATI).

2.4. Adhesion assay

Cover slips were coated with several concentrations of poly(PHG), CRP, or collagen, then blocked with BSA [11]. Washed human platelets were seeded on the cover slips for 30 min at room temperature. After removal of unbound platelets, adherent platelets were fixed, permeabilized, stained, visualized and photographed as described elsewhere [11]. Adherent platelets (0.022 mm^2/image) were counted and mean number of adhesion ± S.E. per image from at least eight different images from two separate experiments was calculated.

2.5. Immunoprecipitation, pull down assay, and Western blotting

Washed platelets were pretreated with 1 mM GRGDS peptide to inhibit the effect of platelet aggregation and then activated with CRP or poly(PHG). Immunoprecipitation and pull down assay were performed as described previously [10]. Briefly, reactions were terminated by the addition of 2× lysis buffer. The lysates were precleared with protein G-Sepharose and insoluble materials were removed by centrifugation. A small aliquot was dissolved with

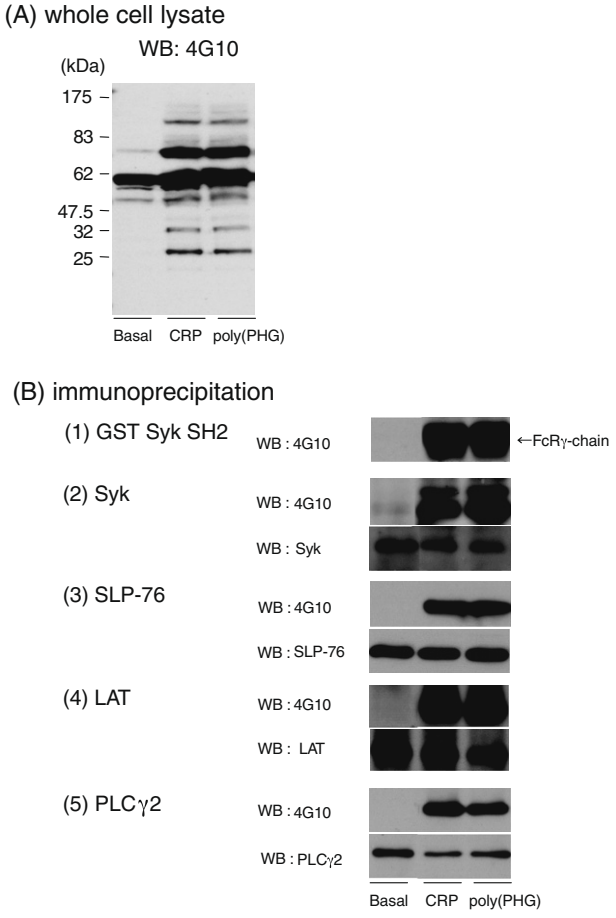

Fig. 2. Poly(PHG) stimulates increase in tyrosine phosphorylation of signaling molecules downstream of GPVI. Human washed platelets were stimulated with 3 μg/ml of CRP or 0.3 μg/ml of poly(PHG) for 2 min. Immunoprecipitation using antibodies against Syk, SLP-76, LAT, or PLCγ2, or pull down assay using GST-Syk SH2 were performed as described in Section 2. Whole-cell lysates (A) or precipitated proteins (B) were separated by SDS–PAGE, electrotransferred, and Western blotted by the indicated antibodies.

SDS sample buffer for detection of total tyrosine phosphorylation. Antibodies against Syk, SLP-76, LAT, or PLCγ2 plus protein G-Sepharose or GST-Syk-SH2 that had been coupled to glutathione-Sepharose were added to the resultant supernatant and incubated overnight. The Sepharose beads were washed before addition of SDS sample buffer. Whole-cell lysates or precipitated proteins were separated by SDS–PAGE, electrotransferred, and Western blotted as described previously [10]. Tyrosine-phosphorylated FcRγ-chain is observed as 10/12 kDa proteins pull-downed with GST-Syk-SH2 [1].

2.6. Surface plasmon resonance spectroscopy

A specific interaction between poly(PHG) and GPVI-hFc2 was analyzed using a BIAcore X (BIAcore AB, Uppsala, Sweden). Poly(PHG) was coupled to a CM5 tip (BIAcore), using Amine Coupling Kit (BIAcore) and blocked as described elsewhere [11]. GPVI-hFc2 in HBS-EP buffer (BIAcore) at several concentrations was perfused over the control surface or an immobilized poly(PHG) surface at a flow rate of 20 μl/min at 25 °C, and the resonance changes were recorded. The response from the poly(PHG) surface was subtracted from that of the control. The dissociation constants (Kd) were determined using BIAevaluation software (BIAcore).

2.7. Statistics

The data were expressed as the means ± S.E. and analyzed with a Student's *t*-test. P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Poly(PHG) strongly stimulates platelet aggregation and increase in protein tyrosine phosphorylation of several signaling molecules

Human washed platelets, human platelet-rich plasma (PRP), or human whole blood were stimulated with the indicated concentrations of collagen, poly(PHG), or the GPVI-specific agonist CRP. Poly(PHG) was capable of inducing aggregation of washed platelets (Fig. 1A) and PRP (Fig. 1B) at concentrations of as low as 1 ng/ml, and at least 10 times more potent than CRP and 100 times more potent than collagen. This relationship was roughly applicable for platelet aggregation in whole blood as the minimum concentrations of an agonist that induces 50% aggregation were 0.0037 μg/ml for poly(PHG), 0.049 μg/ml for CRP, and 0.21 μg/ml for collagen (Fig. 1C). Poly(PHG) supported platelet adhesion in a concentration-dependent manner as well as CRP and collagen (Fig. 1D). Number of adhesion was reached to a plateau at a concentration of 5 μg/ml in poly(PHG), 50 μg/ml in CRP, or 100 μg/ml in collagen

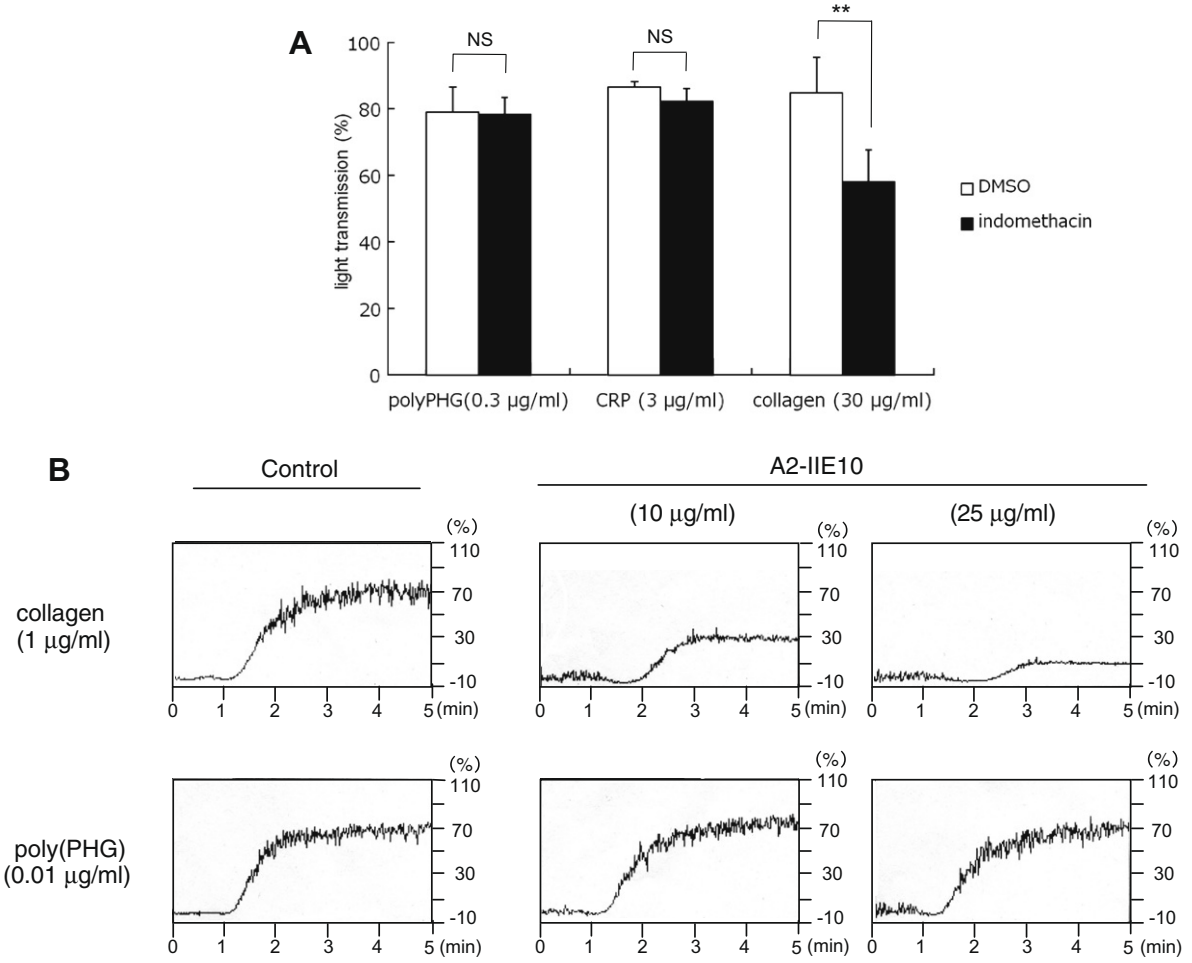

Fig. 3. Poly(PHG) induces platelet aggregation independently of TXA2 or integrin α2β1. Human washed platelets (2×10^8/ml) were pretreated with DMSO or 10 μM indomethacin (A) or anti-integrin α2 antibody (A2-IIE10) (B) and then stimulated with collagen, CRP, or poly(PHG). Platelet aggregation was monitored by an aggregometer for 5 min. The data in (A) are expressed as the means ± S.E. and analyzed with a Student's *t*-test (n = 6–15 from four experiments). Two asterisks denote $P < 0.005$. NS denotes "not significant". The data in (B) are the representative of three experiments from different donors.

(Fig. 1D), which also showed potent platelet activating ability of poly(PHG).

To investigate the molecular basis of platelet aggregation induced by poly(PHG), we examined whether poly(PHG) stimulates protein tyrosine phosphorylation, to which GPVI-induced platelet activation signals are related. It is well known that cross-linking of GPVI elicits a signal transduction cascade that involves sequential activation of Src family tyrosine kinases, phosphorylation of FcRγ-chain and Syk activation, leading to PLCγ2 activation [1,12,13]. The adapter proteins LAT and SLP-76 play a critical role in the regulation of PLCγ2 downstream of Syk [14,15]. Western blotting analysis of whole-cell lysates showed that poly(PHG) induced marked increase in tyrosine phosphorylation of proteins, the pattern of which was almost identical to that observed in CRP-stimulated platelets (Fig. 2A). Immunoprecipitation studies revealed that the proteins that undergo an increase in tyrosine phosphorylation upon platelet stimulation with poly(PHG) are invariably present in the CRP-stimulated GPVI signaling cascade, namely FcRγ-chain, the tyrosine kinase Syk, the adapter proteins LAT and SLP-76, and PLCγ2 (Fig. 2B). These findings imply that poly(PHG) induces platelet aggregation depending on GPVI, similar to CRP.

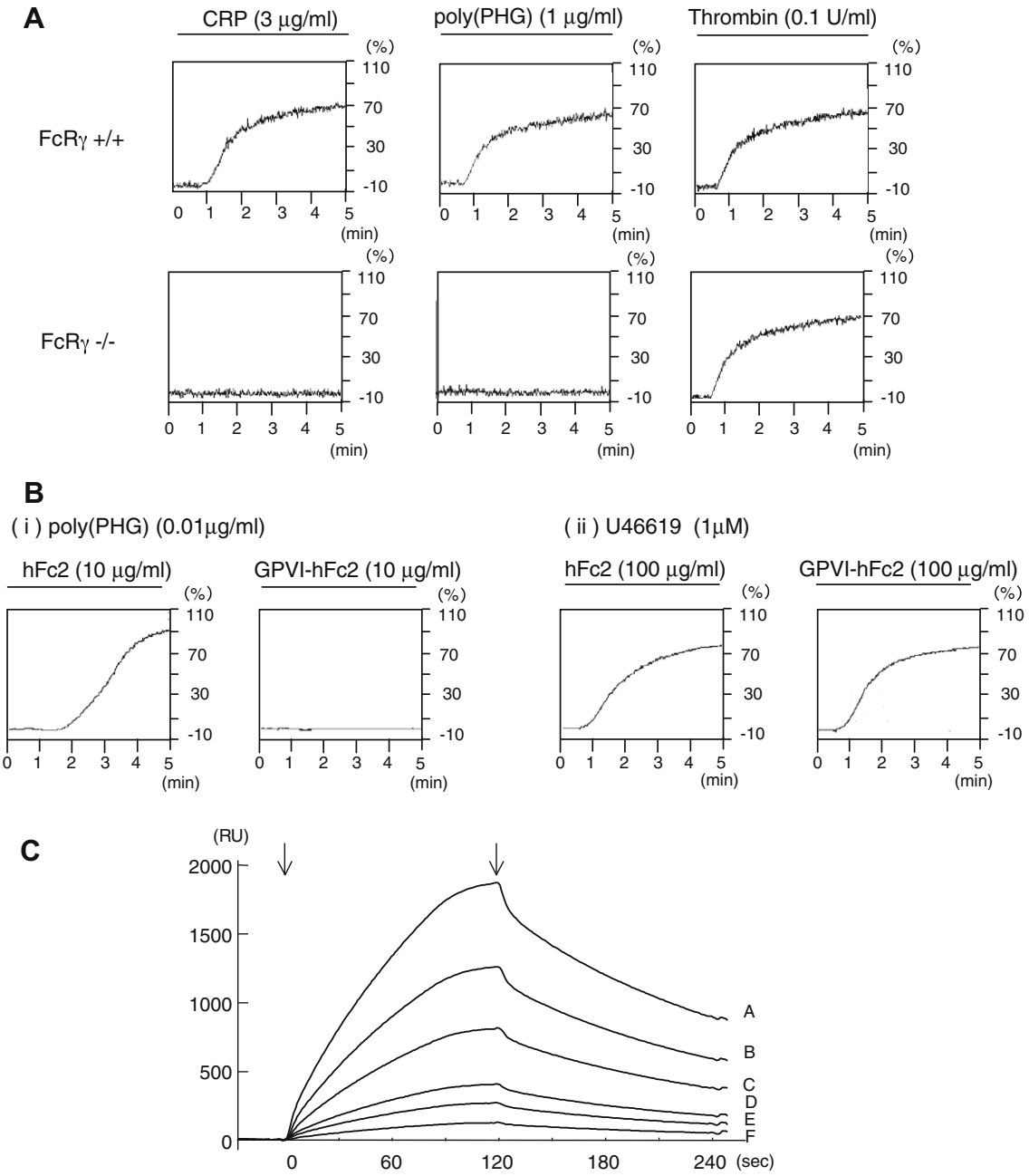

Fig. 4. Poly(PHG) stimulates platelets by binding to GPVI/FcRγ-chain. (A) Murine washed platelets (2×10^8/ml) were stimulated with the indicated concentrations of CRP, poly(PHG), or thrombin and platelet aggregation was monitored by an aggregometer for 5 min. The data are representative of three independent experiments. (B) Washed human platelets were pretreated with indicated concentrations of hFc2 or GPVI-hFc2, then stimulated with 0.01 μg/ml of poly(PHG) (i) or 1 μM U46619 (ii). (C) The specific interaction between GPVI and poly(PHG) was analyzed using BIAcore. GPVI-hFc2 at several concentrations was perfused over a control- and a poly(PHG)-surface of a CM5 sensor tip and the resonance changes were recorded. The arrows indicate the beginning and end of perfusion of GPVI-hFc2. The data are the representative of three experiments. RU: resonance units, A: 500 nM, B: 333 nM, C: 200 nM, D: 133 nM, E: 66.7 nM, F: 33.3 nM.

3.2. Poly(PHG) induces platelet aggregation independently of the secondary mediator TXA2 and integrin α2β1

It is well known that the GPVI agonists CRP and convulxin can activate platelets independently of TXA2; however, responses to collagen were dependent on the secondary mediator [1]. A major difference between platelet activation induced by the GPVI specific agonists and collagen is the involvement of additional receptors for collagen in platelets, most notably the integrin α2β1 [1]. Since natural collagen is a macromolecule and the content of the Gly-Pro-Hyp motif recognizable with GPVI is only 10% of the total weight of collagen fibers, it could not firmly bind to GPVI without the help of α2β1. However, the content of the Gly-Pro-Hyp motif in CRP is 80%, which could firmly bind to GPVI without the help of α2β1. Affinity of α2β1 for their ligands is increased by inside-out signals elicited by the receptors for ADP, TXA2, which promote firm platelet–collagen interactions, leading to enhanced signaling [1].

Platelet aggregation induced even by a supra-maximal concentration of collagen (30 μg/ml) was significantly inhibited by the blockade of TXA2 production by indomethacin, whereas CRP- and poly(PHG)-induced platelet aggregation was not affected by indomethacin (Fig. 3A), suggesting that α2β1 which requires TXA2 production is not involved in poly(PHG)-mediated platelet activation. Fig. 3B shows that the anti-α2 blocking antibody dose-dependently inhibited collagen-induced platelet aggregation, but not that of poly(PHG) (Fig. 3B), further confirming that poly(PHG) stimulates platelet aggregation independently of α2β1.

3.3. Poly(PHG) failed to induce platelet aggregation in the absence of GPVI/FcRγ-chain

To verify that poly(PHG) induces platelet aggregation depending on GPVI, we utilized murine platelets from mice lacking GPVI/FcRγ-chain. Platelet aggregation induced by CRP or poly(PHG), but not that induced by thrombin, was completely inhibited in the absence of GPVI/FcRγ-chain complex (Fig. 4A). Moreover, recombinant extracellular domain of GPVI fused with human IgG Fc2 (GPVI-hFc2), but not recombinant human IgG Fc (hFc2), completely inhibit poly(PHG)-induced platelet aggregation, whereas both recombinant proteins did not affect a TXA2 mimetic U46619-induced platelet aggregation (Fig. 4B). A surface plasmon resonance spectroscopy (BIAcore) confirmed direct and dose-dependent binding of the recombinant GPVI to poly(PHG)-coated surfaces (Fig. 4C). The K_d for the interaction of GPVI-Fc2 with poly(PHG) was calculated as 54.5 ± 43.0 μM (means ± S.D.), that was almost equivalent to that of GPVI-hFc2 with CRP (27.1 ± 19.0 μM) [7]. These data suggest that poly (PHG) indices platelet aggregation solely by binding to GPVI, independently of integrin α2β1.

4. Discussion

We demonstrated that the synthetic collagen poly(PHG) stimulates platelet aggregation by binding to GPVI. One of the issues to be addressed is the difference between the GPVI-specific agonist CRP and our poly(PHG). CRP is comprised of 10 (Gly-Pro-Hyp) motifs and either a lysyl or a cysteine residue at both ends (Gly-Lys(or Cys)-Hyp-(Gly-Pro-Hyp)10-Gly-Lys(or Cys)-Hyp-Gly) that mimic the tertiary (triple-helical) structure of collagen molecules [2]. Since the polymeric structure of collagen is essential for its platelet-stimulating activity, cross-linking via either lysyl or cysteine residues to impart quaternary (polymeric) structure is essential [2]. On the other hand, poly(PHG), as well as natural collagen, spontaneously assemble into a polymeric (organized triple-helical) structure without chemical cross-linking [5]. This may be because CRP consists of only ten (Gly-Pro-Hyp) motifs, which is not long enough to physically form the polymeric structure, but poly(PHG) has long fiber-like structure, which may facilitate spontaneous polymerization. The transmission electron microscopic images of poly(PHG) and CRP in a previous report support this notion; poly(PHG) assumes fiber-like structures [5], while there are micro-aggregates with CRP preparations [2]. Our present data demonstrate that poly(PHG) is at least 10 times more potent than CRP and 100 times more potent than collagen in inducing platelet activation (Fig. 1). Since the content of the Gly-Pro-Hyp motif recognizable with GPVI is approximately 10% of the total weight of the natural collagen, 80% with CRP, and 100% with poly(PHG), poly(PHG) may be able to more effectively cluster GPVI molecules than collagen or CRP.

CRP and convulxin are widely used as GPVI agonists to investigate the GPVI-mediated activation signals in laboratory research. We propose that poly(PHG) can serve as a better GPVI agonist than CRP and convulxin in several aspects. It is frequently observed that different preparations of CRP cross-linked via either lysyl or cysteine residues show variable abilities to induce platelet aggregation. One the other hand, since poly(PHG) spontaneously forms polymeric structure without cross-linking, variability in its activity induced by different levels of cross-linking could be minimized in the case of poly(PHG). Cost-wise, CRP polypeptide is expensive, as it consists of as long as 37 amino acids. As for convulxin, purification of convulxin from crude snake venoms requires relatively difficult techniques and thus commercially-available convulxin preparations are quite expensive. Since poly(PHG) is made by direct polycondensation of Pro-Hyp-Gly tripeptides on a large scale, the cost of poly(PHG) production is considerably less than that of CRP or convulxin.

In clinical settings, platelet aggregation tests are performed to evaluate bleeding disorders or to monitor the effect of anti-platelet drugs. Animal-derived natural collagen is widely used to stimulate platelet-rich plasma as an agonist of platelet activation. Potential contamination of animal-derived collagen with pathogens has recently created a demand for safer resources for collagen, namely recombinant collagen, and there are several commercially-available products of recombinant human collagen. However, they are extremely expensive. The synthetic poly(PHG) meets the demand well, since it induces platelet aggregation and has no pathogen. Moreover, since it can be produced in a large quantity, a large number of poly(PHG) preparations can be supplied as a uniform quality at a relatively low cost, and thus it is most suitable to realize standardization of platelet aggregation tests with the use of the agonist of the same potency for all the users.

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